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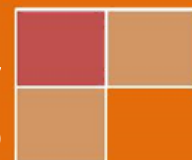
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Uric Acid Biosensor Based on Biofilm of *L. plantarum* using Screen-Printed Carbon Electrode Modified by Magnetite

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Abstract: Biosensor based on biofilm of *L. plantarum* has been successfully done for determination of uric acid in human urine compared with colorimetric enzymatic produced relative error of less than 5%. *L. plantarum* has uricase activity to react with uric acid, to maintain the stability of bacteria forming themselves into biofilms. Magnetite is known to increase sensitivity of the biosensor. The combination of magnetite-polyethylene glycol (Fe₃O₄-PEG) was used to modify the surface of Screen-Printed Carbon Electrode modified (SPCE) and the resulting modified electrode (biofilm/Fe₃O₄/PEG/SPCE) displayed good electrocatalytic activity to the oxidation of UA. The composition of biofilms with optical density 1, magnetite 100 mgmL⁻¹ and PEG 3% v / v were able to increase the current up to 48% in 4mM of UA. The biosensor with an optimum composition produced good linearity with a concentration range, limit of detection, limit of quantitation, sensitivity, and repeatability were found to be 0.1 - 4.3 mM, 70 µM, 234 µM, 25.392 µA mM⁻¹, 2.38%, respectively. This biosensor stable up to 49 days of measurement with the remaining activity was 90.70% and selective for interference compounds such as salt, urea, glucose, ascorbic acid. This method has a good stability, sensitivity, and potential application in clinical analysis.

Keyword: biofilm, biosensor, *L. plantarum*, magnetite, uric acid

1. INTRODUCE

The lifestyle and diet in society are increasingly diverse causing various diseases, one of which is gout. Gout occurs when uric acid levels in excess of the body to form crystals which cause pain in the joints. Uric acid (UA) is the residual metabolism of purine compounds and by-products of the breakdown of cells in the blood. Normal UA levels in the serum and urine excretion are 0.12 to 0.45 mM and 1.4 to 4.4 mM, respectively (Govindasamy, et al., 2016). The development of an early detection technique becomes one of the things considered preventing complications such as cardiovascular disease gout, and kidney failure. Consequently, it is a major interest in research in the field of health. Research that has been done previously to detect UA using chemiluminescence (Chaudhari, et al., 2012), HPLC (Honeychurch, 2017), spectrophotometry (Iswantini, et al., 2009) and colorimetric (Lu, et al., 2017).

Some of these methods have disadvantages such as less specific, complicated preparation, taking a long time in detection and needed experts to operate. In its development, biosensors are one of the detection devices demanded by researchers in an effort to correct the shortcomings of these techniques. A biosensor stands as a measuring instrument utilizing chemical and biological reactions for the detection and quantification of specific analytes based on biomolecules as receptors or their identifying components. Biological identifiers for UA biosensors is uricase enzyme (uric acid oxidase). Uricase enzyme plays a role in catalyzing the uric acid oxidation reaction to allantoin. This enzyme is difficult to isolate from vertebrate animals, expensive and has low stability. Therefore, it is not suitable for long-term use. The use of microbes can be used as another way to obtain enzymes.

Microbes having uricase enzyme are *Pseudomonas aeruginosa* (Anderson and Vijayakumar, 2011), *Gliomastix gueg* (Atalla and Nehad, 2009), and *Lactobacillus plantarum* (Iswantini, 2009).

In this study, *L. plantarum* was chosen as it's easy to obtain, has high survival, not difficult to handle, has uricase specific to UA (Maunatin and Khanifa, 2012); moreover, able to form biofilms (Iswantini, et al., 2016). *L. plantarum* was obtained from the isolation of one of the Indonesian biodiversity, Medan passion fruit skin. It is used by researchers to develop a detection tool for UA due to the uricase activity is 0.0842-0.1073 U/mL (Iswantini, et al., 2009). In planktonic form, bacterial activity and stability are easily disturbed by the external environment. Consequently, bacteria are formed into biofilms, which able to protect cells from environmental changes as the uricase activity for the measurement is maintained so it is suitable for long-term use. Biofilm is a sessile community characterized by cells that are irreversibly attached to a substratum either an interface or to each other, embedded in a matrix of extracellular polymeric substances (EPS) produced, exhibit an altered phenotype with respect to growth rate as well as the gene transcription (Donlan and Costerton, 2002). Biosensor based on biofilm of *L. plantarum* is used as a detector of UA.

The biosensor of UA measurements was carried out using electrochemical techniques. Screen-printed carbon electrode (SPCE) is used as an electrode in this work which has been commercially produced. The analytical performance of the electrode can be increased if modifiers are added which play a role in the accelerating electron transfer (electrocatalytic) and increasing sensitivity of the biosensor. Magnetite is known to deliver electrons directly to the electrode, resulting in increased sensitivity. Nano and micro-sized of magnetite can be combined with disposable electrodes for biosensing platform design (Yáñez-sedeño, et al., 2016). Magnetite has a magnetic force causing inter particles to form a circle (aggregation). Consequently, the magnetite must be stabilized by means of surface functionalization to prevent it. Magnetite can be stabilized by combining steric and electrostatic stabilization by attaching a polymer or organic molecule to the surface of charged particles (Tai, et al., 2016). Polyethylene glycol (PEG) was chosen as a synthetic polymer for magnetite surface modification due to its biocompatibility properties. This study aims are to increase sensitivity and stability the performance of biosensors for the detection of UA in real samples.

2. MATERIALS AND METHOD

Instrumentation

SPCE refs. 110 (DropSens Spain), connector of SPCE DRP-CAC71190 (Metrohm), sonicator (Transsonic 460/H Elma), incubator (Sanyo MIR-162), autoclave (Hirayama HVE 50), centrifuge (5415C), laminar air flow (CVB 1300M), microplate reader (BIO-RAD iMark), pH meter (TOA DK HM-250), scanning electron microscopy (JEOL JSM-6360LA), eDAQ potentiostat (Ecorder 410), x-ray diffraction (Shimadzu 7000), microscope (Nikon YS 100).

Materials

L. plantarum bacterial cells from The Indonesian Research Institute collection isolated from Medan passion fruit skin (*L. plantarum* K. Mar. E), GYP media, uric acid (Nacalai Tesque, Japan), Na₂HPO₄ (Merck), NaH₂PO₄ (Merck), crystal violet 0.02% (Sigma Aldrich), glucose (Merck), 96% ethanol (Merck), ascorbic acid (Merck), FeCl₃ · 6H₂O (Merck), sodium citrate (Merck), urea (Merck), ascorbic acid (Merck), and PEG BM 6000 (Merck).

Procedure

The growth, re-grown, and harvesting of L. plantarum

The growth of *L. plantarum* cells from the main of pure culture into a new pure culture by isolation technique, which is as much as 1 loop of a full culture of *L. plantarum* bacteria scratched on the surface of solid GYP media in petri dishes divided into 4 quadrants with inoculation loops. After that, the petri dish was incubated at 37 ° C for 48 hours. The bacteria that grown then are re-grown by as much as 4 loops of bacterial culture taken and put into an erlenmeyer containing 20 mL of liquid GYP media and incubated at 37 ° C for 24 hours. The bacteria that have grown, centrifuged at a speed of 10 000 rpm for 5 minutes. The formed pellets were washed with 1 mL of phosphate buffer solution (PBS) pH 7 50 mM at eppendorf. The eppendorf is then shaken to be homogeneous and then centrifuged again. Washing with PBS is carried out as much as 3 times repetition. After that, the precipitate is dissolved again in a PBS pH 7 50 mM and then shaken to be homogeneous. A total of 100 µL of bacterial suspension was pipetted and put into a well on a sterile microtiter plate and carried out 2 times repetition. PBS pH 7 is used as a blank. The optical density value of bacteria was measured by microplate reader at a wavelength of 595 nm. The suspension of harvested bacteria is used for biofilm formation on the surface of the electrode.

Preparations of magnetite

Preparations of magnetite referred to Cheng *et al.* (2010) with modification. A magnetite was made from a mixture of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, sodium citrate, and urea with ratio 2: 4: 6 mmol respectively then dissolved in 40 mL of distilled water, stirred until completely dissolved, then put into a Teflon container. The container is put in the oven and set at 200°C for 12 hours. After that, the container is chilled at room temperature. The formed black deposits are separated, washed with water and ethanol, then dried in an oven at 60°C overnight. The synthesized powder was characterized using x-ray diffraction (XRD) and scanning electron microscopy (SEM) to determine the morphology of the synthesis results.

Determination of magnetite and bacterial suspension composites on SPCE

Magnetite is dissolved into 20 µL of PBS, pH 7 50 mM. After that, the mixed solution was dropped directly on the SPCE surface and dried at room temperature for 4 hours. To avoid leakage and immobilization of material on its surface, 3 µL of polyethylene glycol (PEG) is dripped on the surface and dried at room temperature for 2 hours. Suspension of harvested bacteria was dropped 100 µL above the surface of magnetite and PEG composites. Measurements are made after 7 days of bacterial were dropped on the electrode so that bacteria can form biofilms.

Analytical performance

Method validation is measured based on linearity, limit of detection, limit of quantitation, sensitivity, and precision. The linearity from the calibration curve was obtained from 0.1, 0.7, 1.3, 1.9, 2.5, 3.1, 3.7, and 4.3 mM in PBS pH 7 50 mM. Limit of detection (LD) and limit quantitation (LK) were calculated from the standard curve linear regression equation as follows:

$$\text{LD} = 3 \frac{\sigma}{b} \quad \text{LK} = 10 \frac{\sigma}{b}$$

Where s is an estimated blank standard deviation ($n=7$) and S is the slope of the curve. Sensitivity is expressed as the slope of the calibration curve. Method precision is expressed as a percentage of the relative standard deviation (%SBR, $n=7$). Stability is expressed in percent, which is calculated from the oxidation current at day x per day of the initial oxidation current.

Effect of interference for specificity

The selectivity of the UA sensor is evaluated based on the interference of compounds that may be present in the urine. Interference of compounds that normally interfere with UA biosensors includes ascorbic acid (AA), urea (UR), glucose (GL), and salt (ST). Biosensor interference was carried out by comparing the standard PBS pH 7 50 mM with a concentration of AA, UR, and GL 20 times more concentrated than UA.

Application of determination of UA in urine samples

Human urine was collected for 24 hours on the previous day and further quantitative analysis. The samples to be tested with colorimetric enzymatic methods were sent to Prodia, Bogor, which were evaluated as reference methods and compared statistically with the biosensor method.

3. RESULT AND DISCUSSION

Synthesis and characterization of magnetite (Fe_3O_4)

X-ray diffractogram (Fig.1) shows the success of magnetite synthesis, shown by the similarities with the standard pattern, namely the appearance of peaks with the highest intensity at 2θ 35.49 ° close to the standard that appears at 2θ 35.422 (JCPDS 19-0629). The degree of crystallinity of the synthesis results is 67.52% and the average crystal size using the Scherrer equation is 27.43 nM. Thus, crystalline syntheses can be categorized as nanocrystal because the size is less than 100 nM. The shape of the magnetite crystal is a face-centered cube (FCC) as evidenced by the calculation of the crystal lattice parameter, $d = 8.3 \text{ \AA}$.

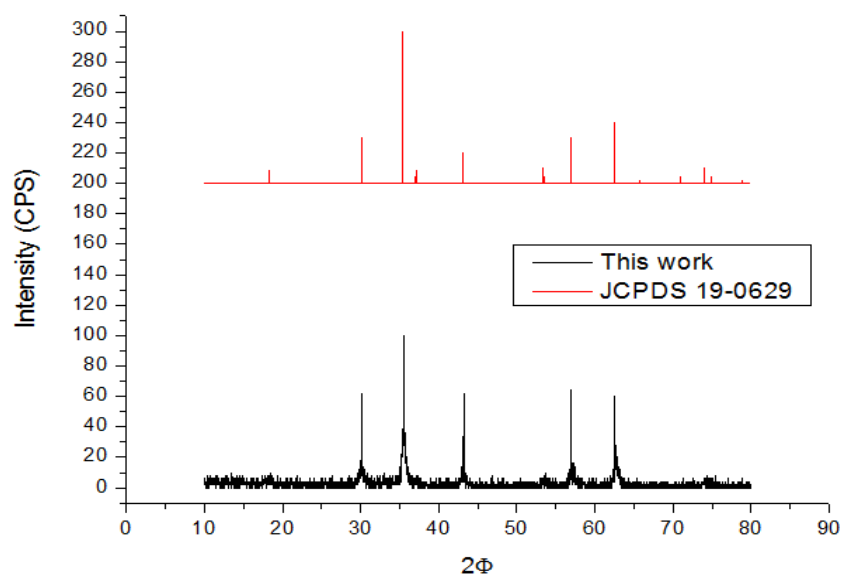


Figure 1. Diffractogram of magnetite synthesis compared to standard

Biofilm activity and Modifier effect as a UA biosensor

The *L. plantarum* bacteria obtained from the isolation of Medan passion fruit skin (*L. plantarum* K. Mar. E cell) were confirmed based on the results of DNA isolation. PlnA gene detection using the PCR method showed a homology level of 99% compared to the Genbank data source (Yulinery and Nurhidayat, 2015). Cell *L. plantarum* K. Mar. E produces the highest uricase activity than *L. plantarum* Mgs cells. Psmb and Mgs. Bst, each isolated from Medan mangosteen and Brastagi mangosteen, which have 0.1073 U / mL of culture (Iswantini *et al.*, 2009). The potential of *L. plantarum* bacteria in forming biofilms is possible. According to Aoudia *et al.* (2016), the duration of biofilm formation in only 24 hours was that the biofilm was flat and homogeneous with an empty hole seen in several strains and produced an optical density (OD₅₉₅) of more than 1 at a wavelength of 595 nm. The OD value of 1 is equal to about 10⁹ cells/mL. The purpose of *L. plantarum* biofilm formation is to increase the activity and stability of the uricase enzyme in the *L. plantarum* bacteria because the activity and stability of the bacteria are easily disturbed by the external environment if it is in planktonic form. The principle of detection of UA is done by measuring the oxidation current resulting from the reaction between the substrate and the enzyme as a bioreceptor or molecular identifier (Fig.2a). The design proposed in this study is shown in Fig 2b. There are three stages of immobilization were carried out. The first stage, the surface of the working electrode from SPCE was dripped with magnetite which was dissolved into a 20 μ L buffer and waited until it was dry. After the first stage is complete, 3 μ L of PEG is dripped above it so that PEG will spread throughout the magnetite surface. The final step is the suspension of bacteria dripped on the SPCE –Fe₃O₄- PEG surface which had been allowed to stand for a day. Testing of UA in PBS pH 7 using the voltammetry method can be done after 7 days of bacteria dosing to ensure that biofilms have formed.

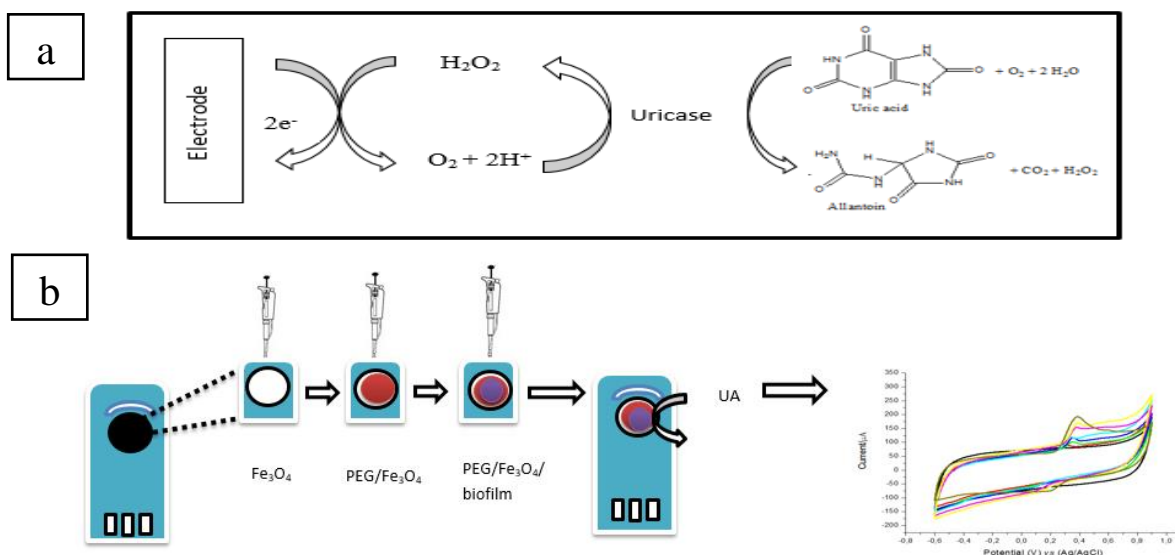


Figure 2. Oxidation of UA catalyzed by uricase enzyme (a) and proposed design of this study (b)

The effect of electrode modification was observed by comparing 4 prepared electrodes: the electrode with magnetite (SPCE-M), the electrode with magnetite and PEG (SPCE-M-PEG), the electrode with bacteria (SPCE-biofilm), the electrode with magnetite, PEG, and bacteria (SPCE-M-PEG-biofilm) with a concentration of 4 mM UA solution in PBS pH 7.50 Mm (Fig.3). Table 1 shows that measurements using magnetite and PEG without bacteria can produce a better current than using the only magnetite with a difference of 7.5 μA . This occurs because of the lack of magnetite properties to adhere well to the electrode. Furthermore, the possibility of magnetite easy aggregation due to the absence of something to resist the pulling force between the particles. Electrodes show the best ability is a combination of biofilm, magnetite and PEG which can increase current up to 48%; 41%; 25%; compared with magnetite, magnetite-PEG, and biofilm alone. There is an increase in flow due to the presence of biological material (uricase) which is able to recognize substrates better; moreover, in the form of biofilms causing magnetite firmly attached to the electrode. These results are expected with the presence of uricase to selectively support the introduction of UA. Magnetite has succeeded in increasing anodic current compared to without the addition of these materials. Magnetite does not interfere with voltage changes in measurement due to the existence of anodic peak at the same voltage change as SPCE without magnetite.

Table 1. Modification influence in the current of the biosensor.

Electrodes configuration*	Current (μA)
SPCE-M	116,9
SPCE-M-PEG	124,4
SPCE-biofilm	139,5
SPCE-M-PEG-biofilm	165,6

*Reaction medium: UA 4 mM in PBS 0.05 molL⁻¹ pH 7.0, 100 mgmL⁻¹ of magnetite, OD 1 of biofilm, and 3% v/v of PEG.

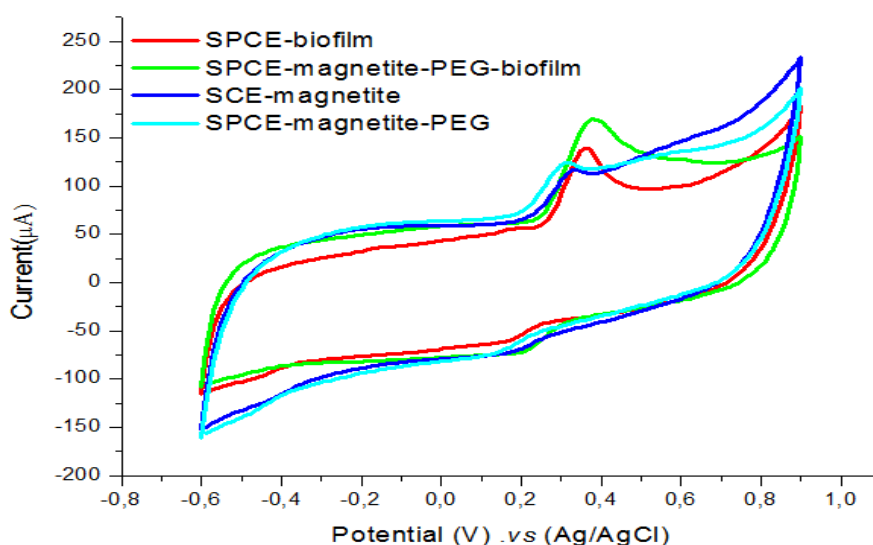


Figure 3. Cyclic voltammogram of modification electrode of the biosensor with a scan rate of 250 mV/s.

Magnetite functions as an electrocatalytic, biocompatibility, and non-toxic activity for the use of biosensors. This is reinforced by magnetite-modified glucose biosensors which show high sensitivity, wide linearity and low detection limits (Sanaeifar, *et al.*, 2017). SEM characterization shows that round and agglomerated magnetite particles are shown in Fig 4a. According to Cheng *et al.*, (2010), agglomeration in magnetite synthesis is very difficult to avoid due to its magnetic properties. Magnetite have strong van der Waals forces and attractions between particles, which can cause magnetite dispersion becoming very unstable and all particles susceptible to aggregation. Tai *et al.*, (2016) on the synthesis results showed that the addition of PEG to the magnetite surface can minimize aggregation by coordination through carbonyl groups and both have dipole-cation binding between the ether group of PEG and the positive charge of magnetite. Efforts to immobilize magnetite, PEG with bacteria produce improved measurements as a result of the magnetite stabilized by PEG and biofilms help to attach the material. The use of polymers can bridge the interaction of bacteria with supporting material when the substrate is added so that the deviation value can be minimized. Biofilm-based uric acid biosensors using SPCE modified magnetite and PEG showed that bacteria that have been attached to the working electrode to form biofilms and magnetite do not interfere or kill bacterial activity due to their biocompatibility (Figure 4b). PEG polymers are chosen because they are biocompatible with magnetite and nontoxic properties for bacteria. Biocompatible properties with magnetite allow for increased magnetic properties because PEG can be adsorbed on the magnetite surface (Junejo and Baykal, 2013). Encapsulation with PEG increases particle dispersibility, chemical stability, and inhibits particle aggregation.

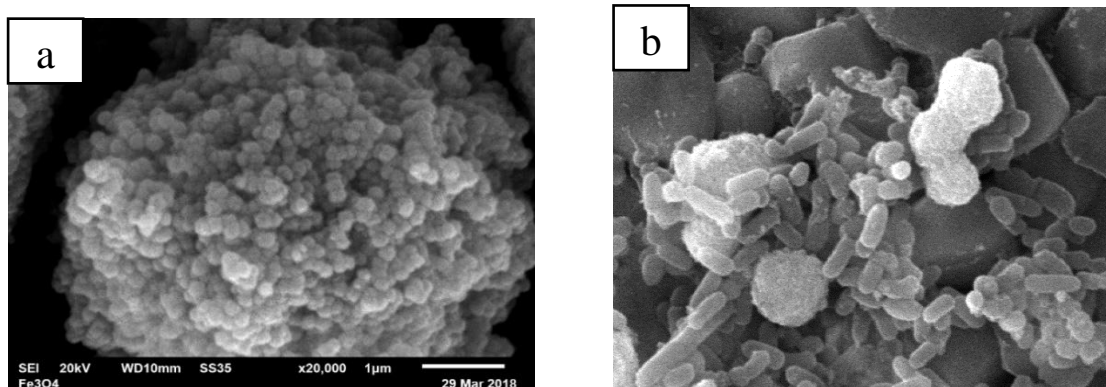


Figure 4. SEM morphology of agglomerated magnetite magnification of 20 000 times (a) and SPCE magnetite-biofilm with 1000 times magnification (b).

Analytical performance

The range of UA concentration used was 0.1 mM to 4.3 mM with an interval of 0.6 mM. The results of linearity (Fig.5a) show that the concentration of UA is directly proportional to the intensity of the peak oxidation current. The equation $y = 25.392x + 71.68$ with the coefficient of determination is 0.99. Anodic peak currents are produced linearly with increasing concentration of analytes.

Based on the results of the study, the LD and LK values for the oxidation reaction were 70 μM and 234 μM , respectively. The sensitivity value obtained is 25.392 $\mu\text{A mM}^{-1}$. Repetition shows favorable value with %SBR is 2.38%.

The maximum UA concentration is 4.3 mM or 0.072%, thus the limit of %SBR value according to AOAC (2011) that less than 1% is 2.7%. Therefore, this biosensor has good accuracy. Stability evaluation showed at the Fig.6b that the *L. plantarum* biofilm immobilized on the SPCE surface remained stable after the 49th day of measurement with the remaining activity of 90.70%. The optimum SPCE biofilm made has a fairly favorable stability with an average value of %SBR that is less than 5%, which is 3.24% at the peak of UA oxidation.

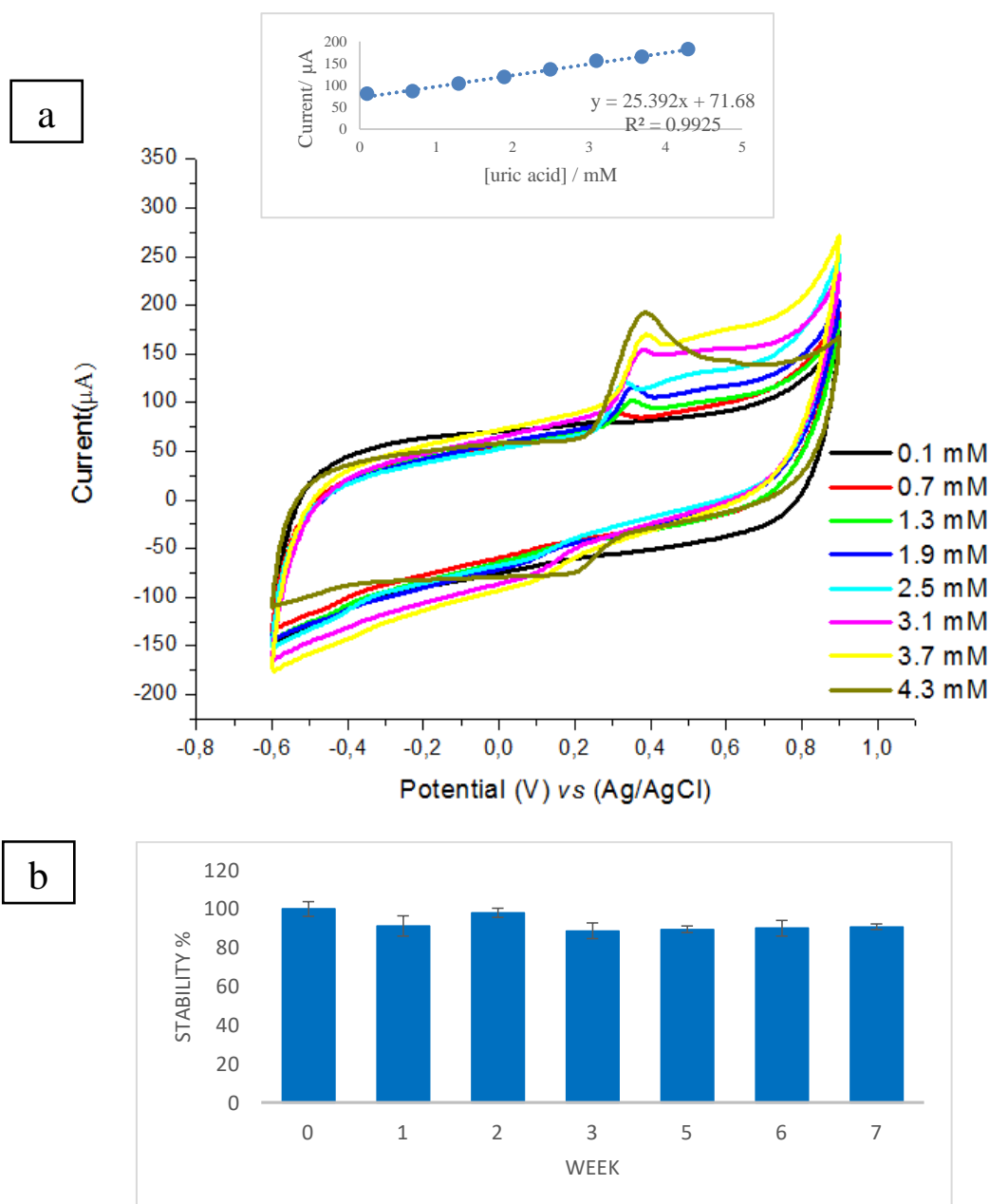


Figure 5. Cyclic voltammogram of linearity with a scan rate of 250 mV/s. Insert: Regression curve of linearity (a) and stability of UA biosensors every 7 days for 49 days (b)

The effect of biological components on the response of the biosensor was evaluated by exposing the biosensor to some potential interferents. The behavior of disturbing compounds that are possible in human urine are glucose, ascorbic acid, urea, and salt. The solution separation method was used in this study on the condition of each compound with a concentration of 20x more concentrated then added UA as the main compound to be measured by comparing the main compound as blank. Responses containing UA showed positive results compared with negative results obtained in samples with UA and disturbing species. Thus, biosensors do not respond to glucose, urea, ascorbic acid or salt (Table 2).

Table 2. Interfering substances (constant uric acid concentration of 0.1 mM) on the voltametry response of the biosensor

Interfering substance	Concentration (mM)	Current (μ A)
Uric acid	0.1	83,5
Uric acid + glucose	2	95,5
Uric acid + ascorbic acid	2	96,7
Uric acid + urea	2	78,3
Uric acid + salt	2	81,9

Application of the Method

The urine samples were collected for 24 hours on the previous day, most of the samples were sent for analysis with colorimetric enzymatic methods while the rest were analyzed by biosensors with 3x repetitions. The results of the analysis of the two methods were statistically tested using the T-test. The results obtained from the method proposed by clinical laboratory methods had no significant difference at 95% confidence level (Table 3). The proposed method can be an alternative solution for detecting UA because it is efficient, easy to prepare, selective, and only requires a small sample. When measuring a sample, the biosensor can be measured and the results are known directly while the colorimetric enzymatic method results can be known after 6 hours from the laboratory.

Table 3. Determination of UA in human urine by reference method and proposed method (n =3)

Sample	Colorimetric enzymatic method (mg/24h)	Biosensor method (mg/24h)	RE (%)
1	165	162.28 (\pm 4.23)	1.65
2	247	246.16 (\pm 3.95)	0.34
3	265	268.34 (\pm 3.97)	1.26

4. CONCLUSION

The combination of magnetite and biofilm is able to make measurements more optimal. Magnetite has been shown to increase sensitivity of analytical performance and biofilms can make enzyme activity are more stable when measurements are maintained. This biosensor has satisfactory linearity, stability, selectivity, repeatability and sensitivity. This tool can also be successfully applied to the determination of uric acid in human urine. In conclusion, the development of biosensors has become a new breakthrough in the methodology of measurement in a practical, fast, sensitive, stable, and selective in determining UA.

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